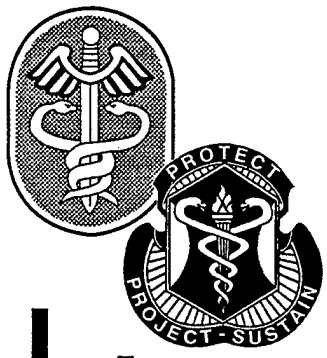

U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE

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Assessment of Edemogenic Effects with Cutaneous Sulfur Mustard Using the Mouse Ear Model

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November 1996

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13. ABSTRACT (*Maximum 200 words*)
Mustard gas (bis(2-chloroethyl) sulfide, HD) is a potent vesicant that rapidly penetrates the skin causing gross and microscopic pathological changes in humans. No definitive animal model produces elevated fluid-filled blisters (bullae) like those seen in human skin. Five dosages of HD (0.08, 0.16, 0.32, 0.64, or 1.28 mg) in methylene chloride ($MeCl_2$) were applied to the inner surface of the right ears of male CD 1 mice (n=10/dosage). Methylene chloride only was applied to the inner surface of the left ears (vehicle control). At 6, 12, 18, and 24 hours post-exposure, 8 mm dermal skin punch specimens were taken from the center of exposed and control ears and weighed to determine fluid accumulation (edema). Skin punches were then fixed in 10 % formalin, embedded in paraffin and stained with hematoxylin/eosin (H&E) for histopathological evaluation. A two way ANOVA on the percent of control edema weight found a significant dose by time interaction, implying that the responses to doses of HD differ with respect to time. Preliminary microscopic examination suggests that sulfur mustard produces tissue damage that is consistent with vesicant injury. Dose and time related edema increases in HD exposed mouse ear skin support the use of this model for studies involving medical countermeasures to cutaneous HD injury.

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PREFACE

The work reported herein was conducted under **U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE** Protocol 1-21-95-000-A-712, entitled "Further Studies of the Mouse Ear Model as an *In Vivo* Bioassay for the Assessment of Topical Sulfur Mustard Injury." The data are recorded in U.S. Medical Research Institute of Chemical Defense notebook 014-95. The work was initiated in April 1995 and completed in July 1995.
A portion of this work was presented in abstract and in a poster session (Assessment of cutaneous sulfur mustard injury in the mouse ear model by Casillas, R.P., Smith, K.J., Reid, T.M., Castrejon, L.R and Stemler, F.W.) at Society of Toxicology, Anaheim, CA, March 1996.

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INTRODUCTION

Mouse ear assays are useful models for studying cutaneous irritant and allergic inflammation (Bouclier et al., 1990; Carlson et al., 1985; Crummey et al., 1987; Patrick et al., 1987). These assays allow for the quantitative assessment of biological and biochemical processes following a chemically induced cutaneous response, and for evaluating pharmacological agents against skin damage. Development of the mouse ear model as a suitable model of cutaneous sulfur mustard injury with potential for screening antivesicants was initiated at the USAMRICD by Brinkley et al. (1989). This current study was undertaken to determine the time course of edema formation in the mouse ear over a broader range of HD dosages in order to complement the findings in the studies of Brinkley et al. (1989 and 1995) and to identify a dose response relationship for evaluating therapeutic compounds.

MATERIALS AND METHODS

Experimental Design and General Procedures: The effect of topical application of sulfur mustard to the inner surface of the right ear was evaluated in albino male mice (Charles River CD 1 Strain) weighing 25-35 grams. The mice were maintained under an American Association for Accreditation of Laboratory Animal Care (AAALAC) program. During quarantine the animals were housed 10 per polycarbonate cage on contact bedding which was changed twice weekly. The mice were provided commercial certified rodent ration as appropriate and tap water *ad libitum.* Animal holding rooms were maintained at 21° ± 2°C with 50% ± 10 % relative humidity using at least 10 complete air changes per hour of 100% conditioned fresh air. All mice were on a 12-hour light/dark, full-spectrum lighting cycle with no twilight.

Standard solutions with 12.5, 25, 50, 100 and 200 μl/ml of neat HD (d = 1.27 g/ml; M W 159; purity 97.5%) in methylene chloride were prepared. The respective standard solutions were equivalent to 0.08 (0.5), 0.16 (1.0), 0.32 (2.0), 0.64 (4.0), and 1.28 (8.0) mg (μmoles) of HD/5 μl aliquot. A 5.0 μl aliquot of the respective solutions was applied topically to the right ear of animals (10 mice/group) with a digital microliter positive displacement pipette (Drummond Scientific Co., Catalog No. 510, Broomall, PA). Due to the volatility of methylene chloride the bottle containing HD solution was immediately recapped following removal of an aliquot. A 5.0 μl aliquot of methylene chloride only was placed on the left ear as control. For comparative purposes, a group of 12 mice received 5 μl of methylene chloride only (six on right ear and six on left) while the opposite ear received no fluid. The latter group was euthanized at the end of 24 hours and evaluated for effects of vehicle on edema.

The mice were weighed and anesthetized with ketamine (57.1 mg/kg) and xylazine (11.4 mg/kg) administered as a single injection intraperitoneally. The mice were placed in a dorsal recumbency on a 4" by 6" cardboard pad with strips of tape over the two front legs and the tail. A large cotton swab (Texwipe) beneath the head was rotated to position the cupped right ear in a horizontal plane. Following evaporation of applied solutions, two mice were housed together in a

a polycarbonate container in the hood. Each container, resting on a pad with circulating heated water, was covered with a plastic backed paper diaper to protect the animals from draft.

All animals were kept in the hood until euthanized in a chamber containing halothane at 6, 12, 18, or 24 hours post-exposure. Both ears were removed and a circular 8-mm punched specimen from each ear was placed in a tared vial and was weighed on an analytical balance (Mettler-Toledo Co., Model A0104, Switzerland).

Edema was expressed as percent of control according to the following equation:

$$\frac{\text{weight right ear (exposed) - weight left ear (control)}}{\text{weight left ear (control)}} \times 100$$

Statistical analysis.

A two way ANOVA was used to compare weights of left versus right ears and weights of untreated ears (no $MeCl_2$) versus $MeCl_2$ treated ears. A two way ANOVA was used to assess dose by time interactions. The amount of edema following each dose was compared using percent of control edema. Statistical significance was defined as $p < .05$. A significant dose by time interaction was observed. Therefore, one way ANOVAS were used to analyze dose differences at each observation time followed by Newman-Keuls tests to assess individual dose differences.

RESULTS

The weights of methylene chloride only treated ears (right or left) and contralateral untreated ears are shown in Figure 1. No significant differences ($p < .05$) in ear weights were observed for untreated (no $MeCl_2$) versus $MeCl_2$ treated ears and in left versus right ear.

The weights of control left ($MeCl_2$ only) and right ears at the five dosages of HD are shown in Figures 2, 3, 4, and 5. The percent of control was calculated for edema and used to compare the doses at each observation. At all time points, the three lower doses, 0.08, 0.16, 0.32 mg HD, had significantly lower percent of control edema ($p < .05$) than the 0.64 mg HD dose. At 6-, 12- and 24-hour time points, the three lower doses had significantly lower percent of control edema than the 1.28 mg HD dose, $p < .05$. In addition, at all time points, the 0.08 mg HD dose had a significantly lower edema effect than the 0.32 mg HD dose, $p < .05$.

The initial localized effect of HD, which begins as an erythema, was visibly obvious in all animals within 30 minutes. Subsequent observations of left and right ears revealed a progressive thickening and swelling of the right ear. Fluid accumulation as measured by progressive weight increases was observed to continue throughout the periods of measurement from six to 24 hours. Histological evaluation is ongoing and will be reported when completed.

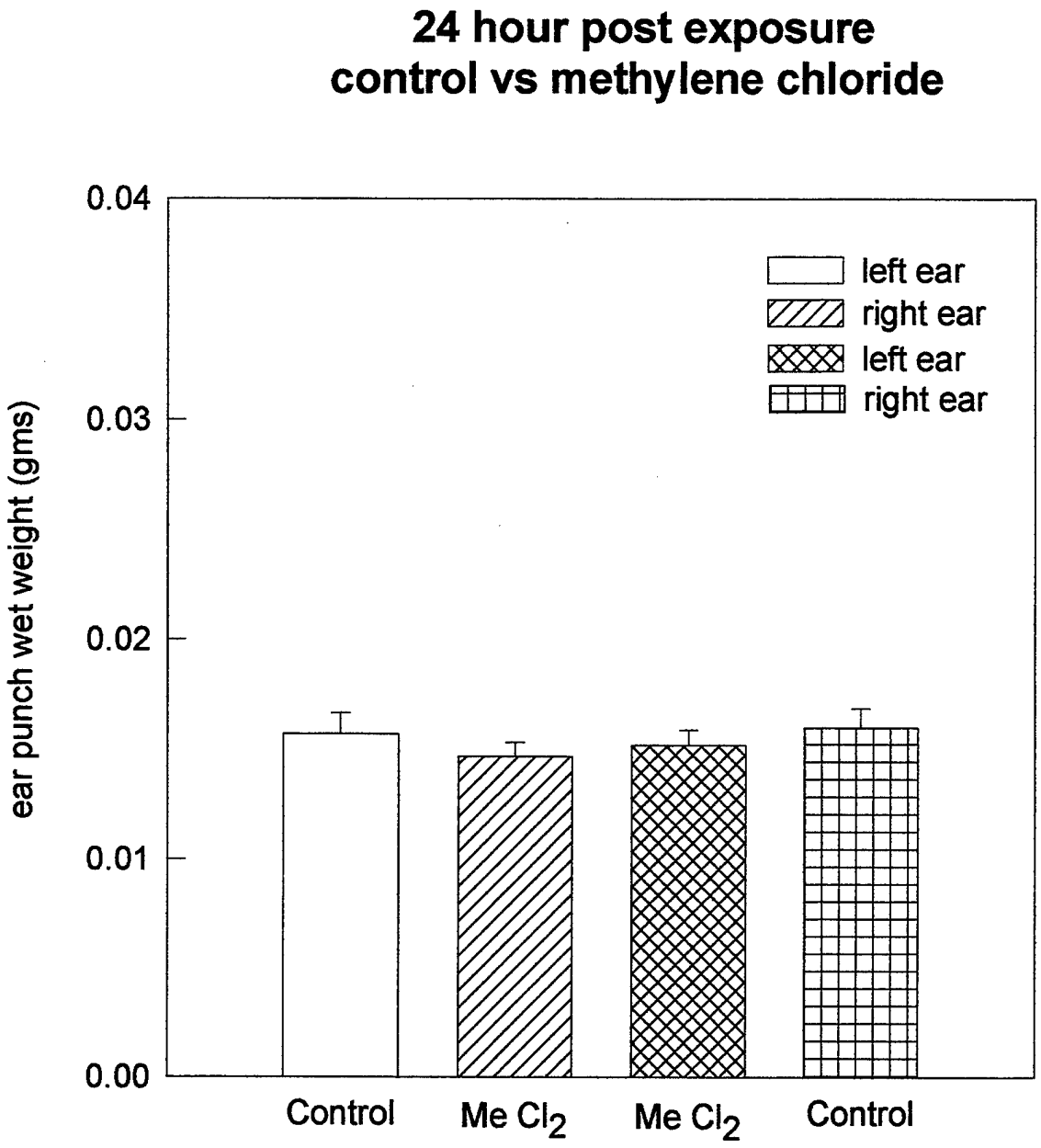

Figure 1 shows the mean weights of left and right ears of mice (N = 6/group) in which 5 μl methylene chloride only was applied to one ear, while the opposing ear served as control. Error bars = ± SEM. $p < .05$.

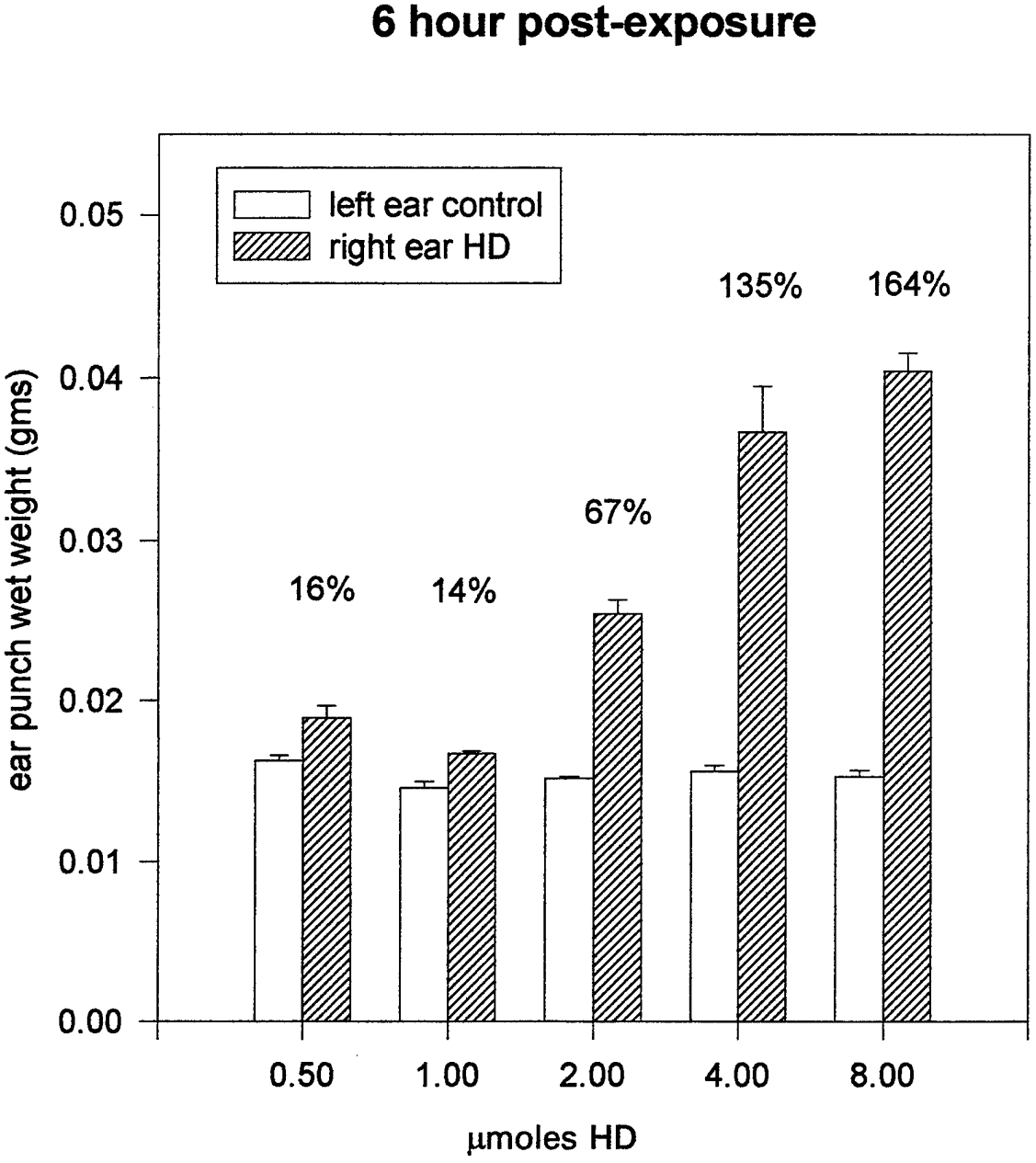

Figure 2 shows the mean weights at 6 hours post-exposure of left methylene chloride treated ears and after topical application of 5 µl aliquots of HD on the right ears of mice (N = 10/group). Error bars = ± SEM. $p < .05$. Numbers refer to % of control.

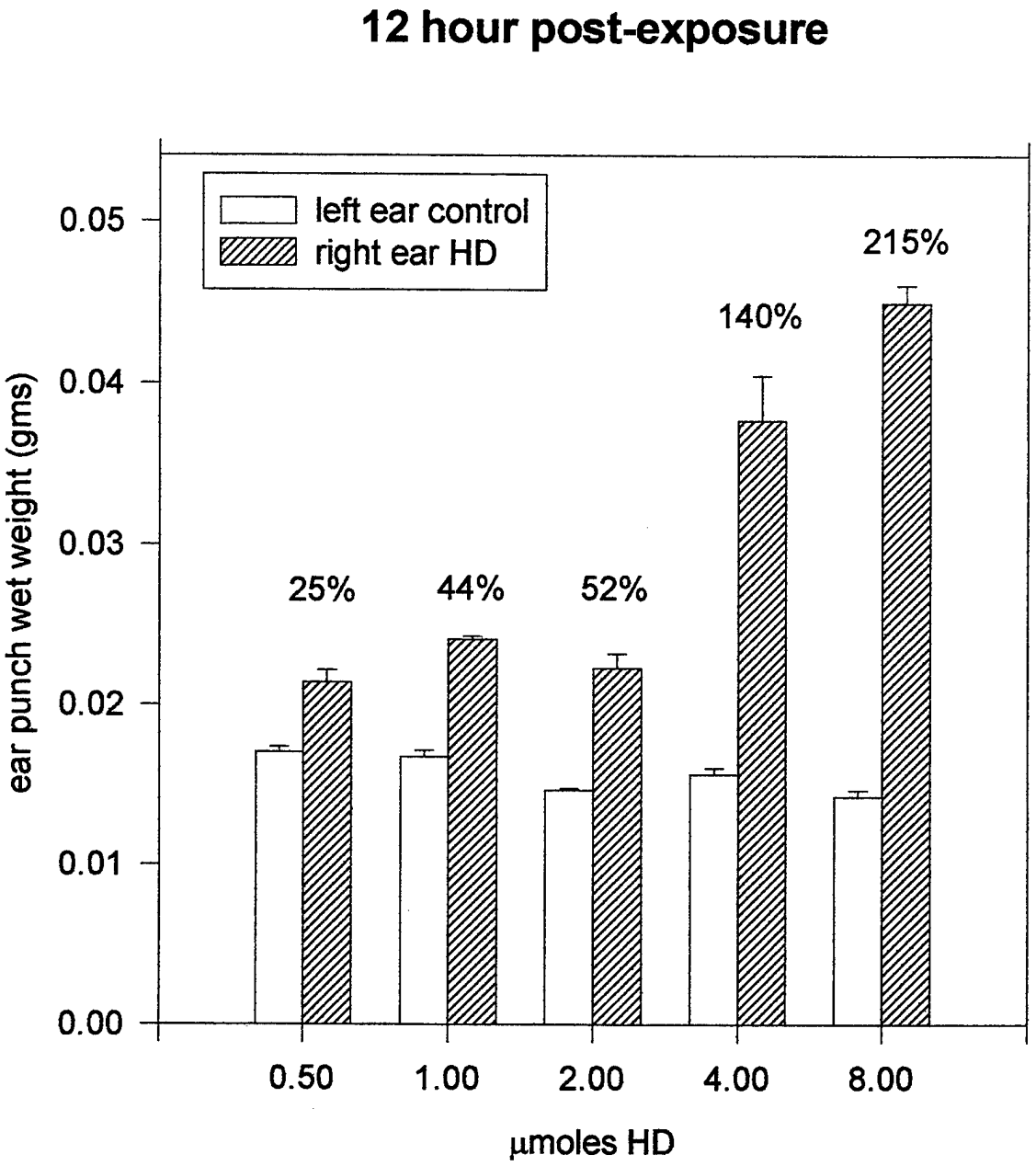

Figure 3 shows the mean weights at 12 hours post-exposure of left methylene chloride treated ears and after topical application of 5 µl aliquots of HD on the right ears of mice (N = 10/group). Error bars = ± SEM. $p < .05$. Numbers refer to % of control.

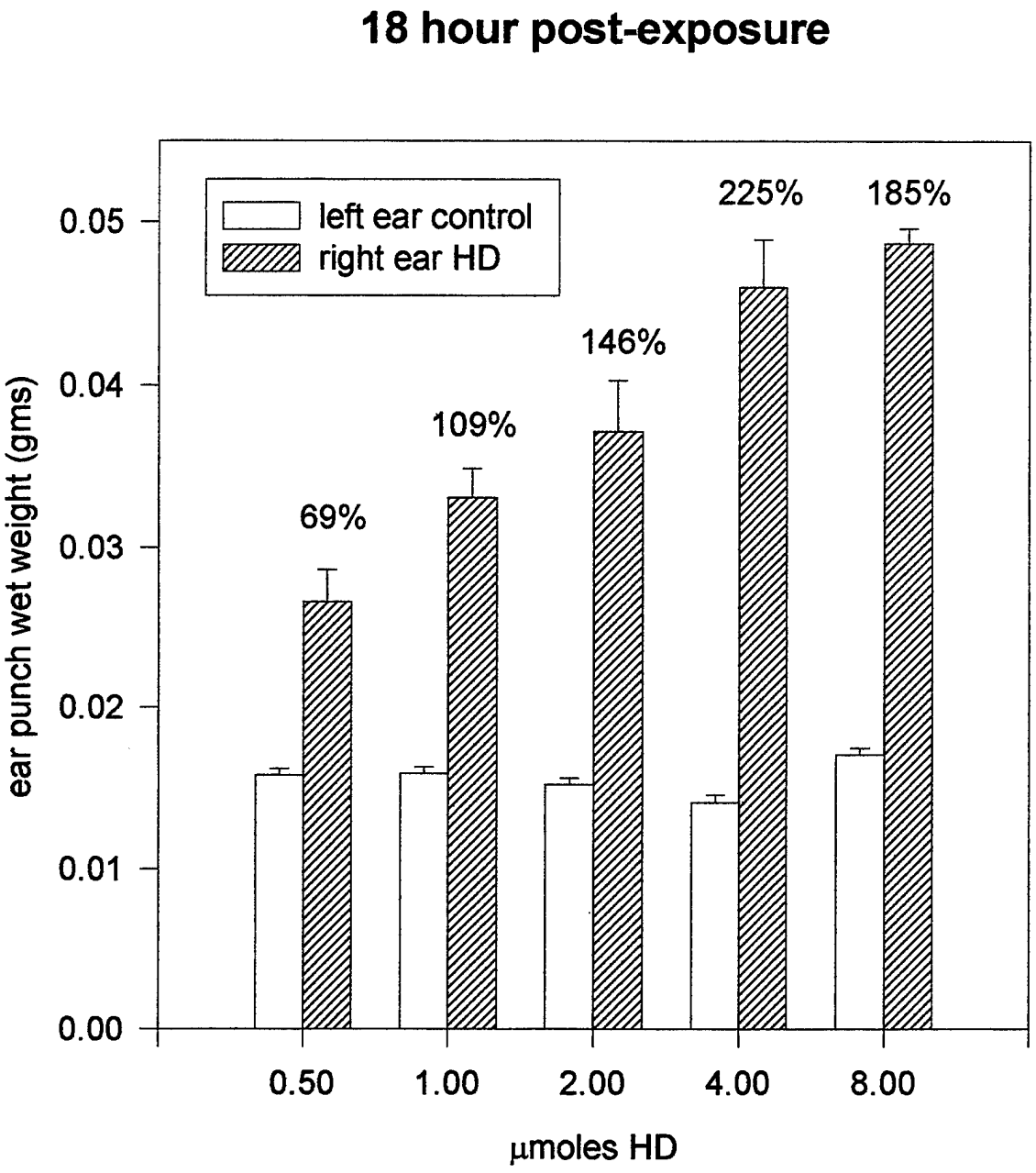

Figure 4 shows the mean weights at 18 hours post-exposure of left methylene chloride treated ears and after topical application of 5 µl aliquots of HD on the right ears of mice (N = 10/group). Error bars = ± SEM. $p < .05$. Numbers refer to % of control.

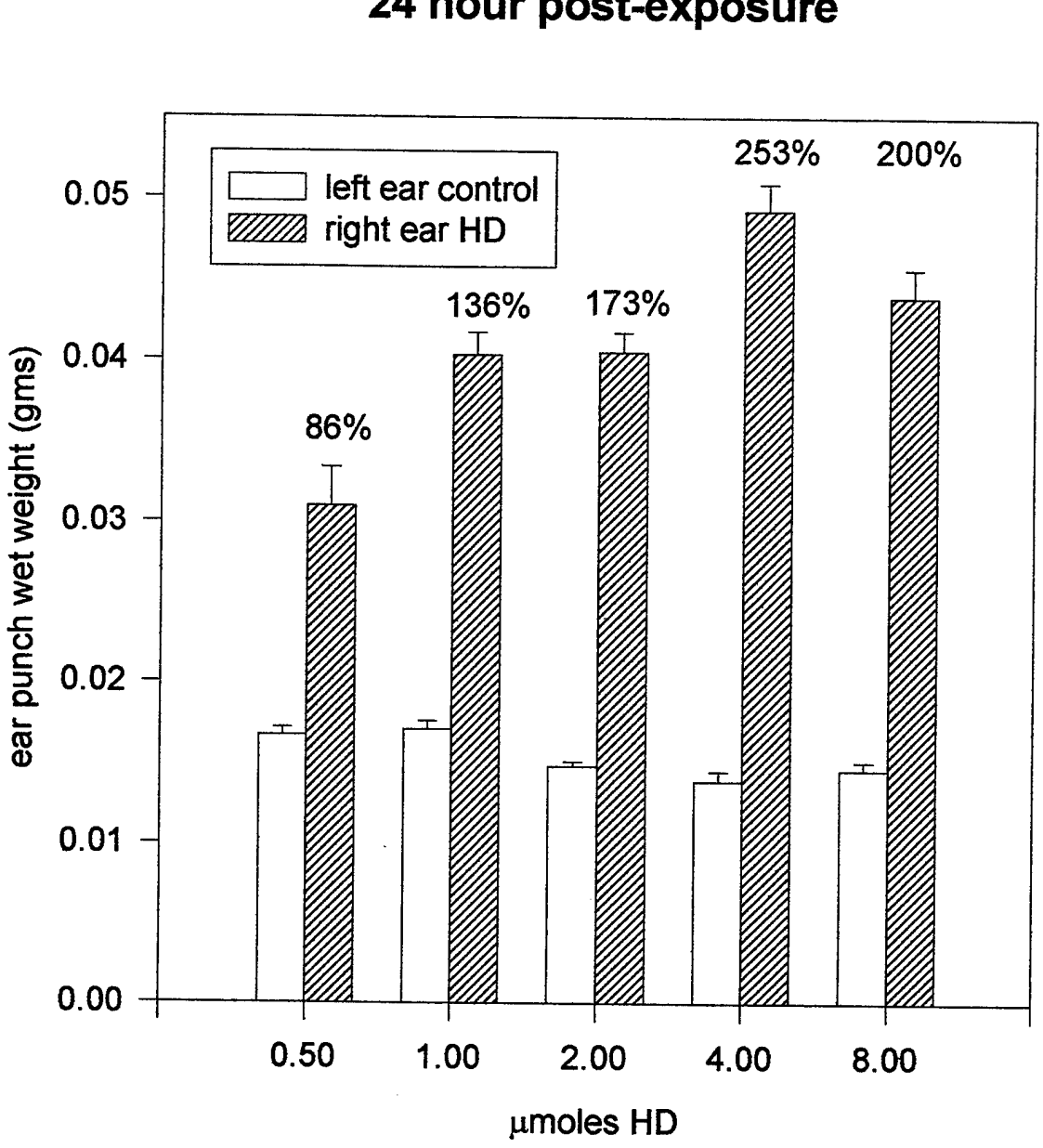

Figure 5 shows the mean weights at 24 hours post-exposure of left methylene chloride treated ears and after topical application of 5 µl aliquots of HD on the right ears of mice (N = 10/group). Error bars = ± SEM. $p < .05$. Numbers refer to % of control.

DISCUSSION

The prolonged time course of fluid accumulation observed following topical cutaneous application to HD on the mouse ear differs from the time course of edema seen with other irritants in this same model (Patrick et al., 1987). Inflammatory responses by measurements of ear weight or ear thickness were compared, and the reponses showed that the time course varied considerably with the irritant. Maximal edema responses occurred in a range of minutes to hours. The prolonged duration of edema in response to HD in the mouse ear model contrasts sharply with the course of edema produced by two commonly used irritants, 12-0-tetradecanoylphorbol acetate (TPA) and arachidonic acid (AA) (Young et al., 1983). The application of TPA to the mouse ear produced a maximal edema response by 6 hours which was decreased substantially by 24 hours. With AA a maximal edema response was reported by 1 hour which decreased substantially by 6 hours. In contrast, fluid accumulation in HD exposed ears at three low dosages (0.08, 0.16 and 0.32 mg HD) progressed throughout the 24-hour period of observation. However, at two higher dosages (0.64 and 1.28 mg HD) fluid accumulation was accelerated and appeared to reach a maximal edema response at six hours. The skin from the mouse ear does develop initial erythema and edema followed by eventual separation of the epidermal-dermal junction to form microblisters which are consistent with HD blister formation in humans. HD differs also from the two above mentioned irritants with the delayed formation of microblisters.

Erythema is due to arteriolar vasodilation. However, the leakage of vascular protein and fluid into the extravascular tissue space is a more complex event involving the interaction of many physiologic factors. These factors include permeability changes in the vascular endothelial lining, mediator release, blood flow changes and/or accumulation of leukocytes (Patrick et al., 1985). The initial formation of tissue edema is due to leakage of proteins through postcapillary venules (Sedgwick and Willoughby, 1985).

It is unknown whether all chemical irritants produce inflammatory responses via the same mechanisms. A comparative study of reported tissue weight or ear thickness in response to several irritants applied to the mouse ear suggested that chemicals produce skin irritation by multiple mechanisms (Patrick et al., 1985). Microscopic examination of tissue lesions showed that each chemical differed in the time course of edema, vessel dilation, cellular infiltrates, permeability responses to dyes and ear temperatures. The latter is an indirect estimate of blood flow. Currently, there are several hypotheses which attempt to explain the mechanism(s) underlying irritant effects of HD (Papirmeister et al., 1991).

Despite a better basic understanding of the mechanisms of sulfur mustard injury, relatively few new therapeutic advances in treatment of HD injury have been made in the past fifty years. The rapidity of cutaneous HD penetration and resultant physiological damage have placed limitations on therapeutic options. Thus, the HD mouse ear model may be a valuable tool for evaluating and screening pretreatment pharmacologic compounds for anti-inflammatory effects, for

antiedemogenic activity, and/or possible inhibition of microvesication. Comparisons of the effectiveness of anti-inflammatory compounds against HD and other irritants in the mouse ear model may also provide insights into the mechanism(s) underlying inflammatory responses.

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